Cultured Neuron Probe

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This QPR is being sent to
you before it has been
reviewed by the staff of the
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General Introduction

Our aim is to develop a cultured neuron probe. This consists of a silicon structure into which individual dissociated neurons can be placed, and which can be inserted into an intact nervous system. Furthermore, within the structure each neuron is in close proximity to an electrode, by means of which it can be stimulated, or its activity can be recorded, through electrical leads which connect to external electronics. It is hoped that neurons in the probe will synaptically integrate with the host nervous system, to provide a highly specific, relatively non-invasive, two-way communication channel. If this occurs, the methodology has important possibilities for neural prostheses. The goal of this project is to perform initial experiments to establish the feasibility of communication by means of a neuron probe. The tissue we have chosen in which to initially implant the probe is the rat hippocampus. If initial studies are successful, probes will be designed and implanted for communication with sensorimotor cortex.

The neuron probe we plan to fabricate will be made of micromachined silicon and will have sixteen electrodes: one conventional electrode to detect activity during placement of the probe, and fifteen within wells into which neurons will be placed. Its configuration will be similar to that of passive multielectrode probes which have been developed. It will be implanted when the cultured neurons are very young, and after a time of weeks it is hoped that they will have survived and made two-way synaptic connections. By stimulation and recording in the host and probe, we will test for the existence of such connections. An essential feature of the experiments is that the viability of the implanted neurons will be independently determined over time by stimulating them and recording their resulting action potentials.

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Fabrication

At the end of the last quarter a final canopy well design for chips and probes was created, along with the necessary masks. However, during most of this quarter the production of chips and probes was delayed while the microfabrication facility was moved and also while various equipment problems were dealt with.

First to be produced were a small number of successful dummy neuroprobes which are being used on slices here at Caltech. Several wafers have still to be brought to completion. Their processing has been delayed due to the moving of several key pieces of equipment into our new micromachining cleanroom. When the equipment has been brought back on-line, the next set of dummy probes that are finished will be sent to Rutgers for implantation work.

In parallel to the production of the probes, a run of fully functional canopy neurochips has been completed. Refinements of the canopy process has made the fabrication much more robust as compared to the initial runs which produced the first dummy canopy chips. Subsequent runs should show further improvements in the production and result in better yields. A small number of chips which were produced are sufficient to allow electrophyisioligical, *in vitro* experiments to be conducted with the new canopy design.

The start of a new run of canopy neurochips has been delayed because the delivery of an order for the required epi-wafers did not take place after many months of waiting. That problem has apparently been dealt with, and wafers are due in a week or two.

Neurochip Experiments

Near the end of the quarter we obtained a small number of full neurochips with the canopy grillwork design. The chips were designed to have eight wells with each of two tunnel widths, 5 and 10 microns. When mounted, these chips had excellent electrical characteristics, with 40-50 pF shunt capacitance at 1 kHz, and platinized electrode capacitances of 1500-2000 pF. We loaded the wells of several chips with SCG neurons, and outgrowth was similar to that on the dummy canopy chips, with approximately half the wells retaining a growing neuron. The figure below shows very nice outgrowth from one of these chips after one day in culture.
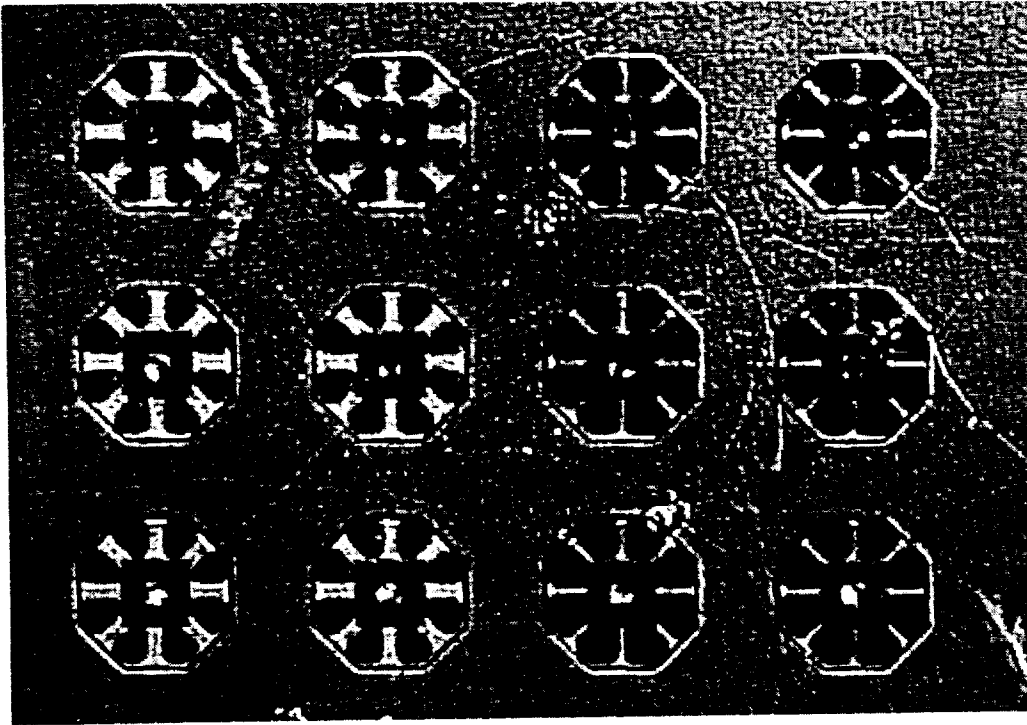
Unfortunately, we encountered a new problem. The connection to the electrodes failed after 2-10 days in the incubator. This problem was traced to the bonding pads on the printed circuit board, where gold wires from the gold-plated pads on the chip were attached with gold epoxy. The contact between the epoxy and the tinned pads of the board became open-circuited as the tinned coating decomposed to an insulating powder. This corrosion took place in the humid atmosphere of the incubator, although the connections and pads were well covered and sealed with silicone and Silastic. Apparently, in the

continuous presence of water, the bond between the Silastic and the printed circuit board weakens, allowing trace amounts of water to reach the tinned contact areas. The weakest point, which is a joint between gold conducting epoxy and the tinned bond pads, always failed first. Corrosion at the interface caused a non-conducting oxide powder to form under the epoxy.

We stripped the tinned pads to bare copper on several PC boards, and had one of them gold-plated. We tested each individual electrical joint in the incubator for 10 days, to determine an acceptable combination. Bare copper or tinned pads with gold epoxy inevitably failed. Gold epoxy on gold plate and on evaporated gold survived with no increase in impedance, as long as the joints were covered with Silastic. (Gold epoxy remains strong even when soaked in warm saline for several days, but the bond to metals (even gold) eventually fails.) The connection of the leads to the evaporated gold pads of the chip with gold epoxy also does not deteriorate when under silastic.

We tested the electrical characteristics of neurochip wells by injecting current from a pipette electrode into the wells of active electrodes. The noise level was 10 μV, and the detected pulse from 1 nA injected into the wells was about 90 μV, so that we should be able to cleanly detect the firing of an action potential in the well. There was no detectable crosstalk between the wells. We plan to use an extracellular stimulating pipette near an axon of a cell in a well. We can reliably stimulate SCG neurons in this manner, with a sharp stimulus threshold of less than 10 μA.

We would prefer to find a potting system which would completely seal the electrical joints from water. At the moment, such a system eludes us, but we now have a bonding system which appears to be stable in the presence of the trace amounts of water that seep through the silicone/Silastic potting. We hope to attempt to record from neurons in the neurochip in the next quarter, and also to stimulate them.

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<u>*In vivo* studies</u>

In many previous attempts we failed to find reliable outgrowth of neuronal processes from silicon probes transplanted into the rat's brain. One of the possible reasons was that hippocampal cells are probably too sensitive to changes of the external environment that occurs during transplantation. We also described that a thin fibrin "film" covered the surface of the probe after we removed it form the brain. The film may stick to the surface of the probe very hard and may block the normal nutrition process and leads to the cell death.

The goal of the present set of experiments was to use septal, mostly cholinergic, cells for transplantation. It is known from experiments with transplantation of cholinergic cells that they survive better during transplantation process than glutamatergic neurons. In addition, their main axon arborizes immediately after emerging from the cell body, therefore may better anchor the cells in the wells. In the present experiments we combined culturing of hippocampal and septal cells. Hippocampal cells were plated around the silicon probes and Dil-stained septal cells were implanted into the wells.

Methods

The hippocampal cells were taken from embryos at E17, and septal cells were taken from embryos at E-15.

The preparation of stained cell suspension and loading them into wells was the same as in previous experiments. The 1×10^6 - 2×10^6 unstained hippocampal cells suspension was plated in each petri dish around the probe. After 1-2 hours of culture the cells that were on the surface of the probe and within wells were sucked away. Stained septal cells were plated within each well. After 24-48 hours of culturing they were transplanted into rats brain for only 3-5 minutes. The intent of these experiments was to determine whether the process of implantation allowed neural survival immediately therafter.

The adult rats were deeply anesthetized, the scalp was opened, the brain meningies were removed by scissors, and the surface of the brain was covered by saline. The part of the probes with the neurons was placed into the

brain with the stereotax. Then, after insertion and removal, probes were placed into a dish with tissue culture medium back and culturing was continued. The examination of outgrowth was done 48 hour later.

Results

Twelve probes with 16 loaded cells each were used for experiments. Six were inserted for 3-5 minutes and 6 were controls. About 70-90% of the cells remained within the wells after 24-48 hours of culturing. About 40-80% of them made processes . The figure below shows outgrowth from a control probe. To date, this was our most successful series in terms of culturing. It is not quite clear whether it has to do with our increasing ability to load cells faster or it has to do with the fact that they were septal cells as opposed to hippocampal neurons used in past experiments. In addition to the loaded cells, about 10-30 cells were left on the surface of the silicon probe and the handle area. Most of them grew processes. Hippocampal cells that were plated around the probes also grew up and made processes up to 14-16 days (maximal length of observation).
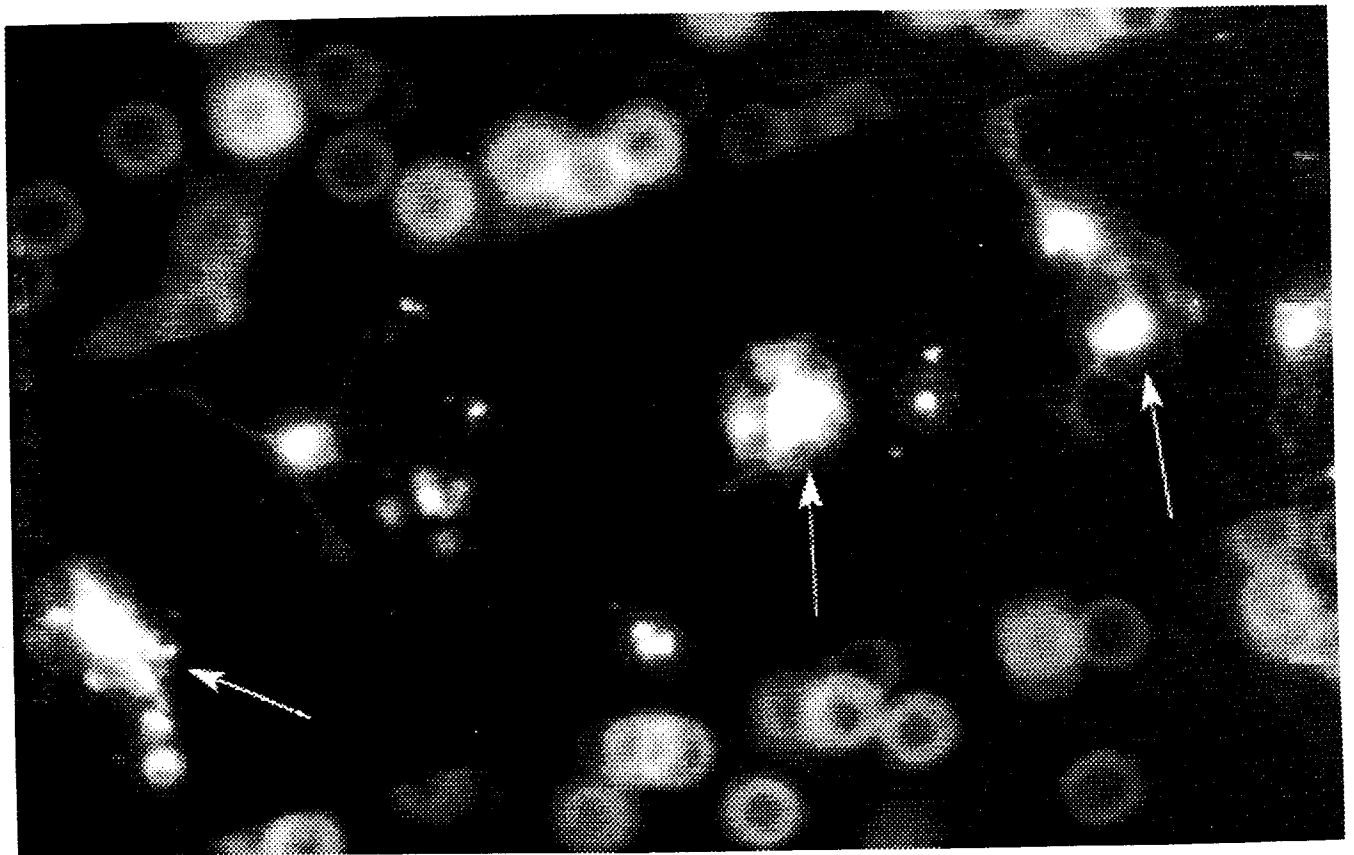
Six probes were inserted into the rat brain for 3-5 minutes. The remaining six were used as controls (Table 1).

Table 1

| Probe No.	No. of neurons in the wells after plating	Hours of 1st culture	No. of neurons remained in the wells after 1st culturing	No. of growing neurons after 1st culturing	No. neurons remaining within the wells after transplantation and 2nd culturing	No. of growing neurons after transplantation and 2nd culturing
DP-41	16	24	11	2	7	no
DP-32	16	24	15	1	8	no
DP-39	16	24	16	2	control	5
2-7-A	16	24	12	3	control	9
2-7-B	16	24	16	3	control	13
2-7-C	16	24	15	2	3	no
2-7-D	16	24	14	1	10	no
2-13-A	16	24	13	3	9	no
2-13-B	16	24	11	2	control	7
2-13-C	16	24	9	1	8	no
2-13-D	16	24	10	3	control	8
2-13-E	16	24	8	0	control	3

As in previous experiments, a thin film covering the surface of the probe was observed after the transplantation. There was no growth of cells within wells. We were able to see the round shape cells through the film at 24-48 hours. It is very likely that they died since DiI stained round shapes were typically seen in the wells of the probe, as shown in wells labelled with arrows in the figure below.

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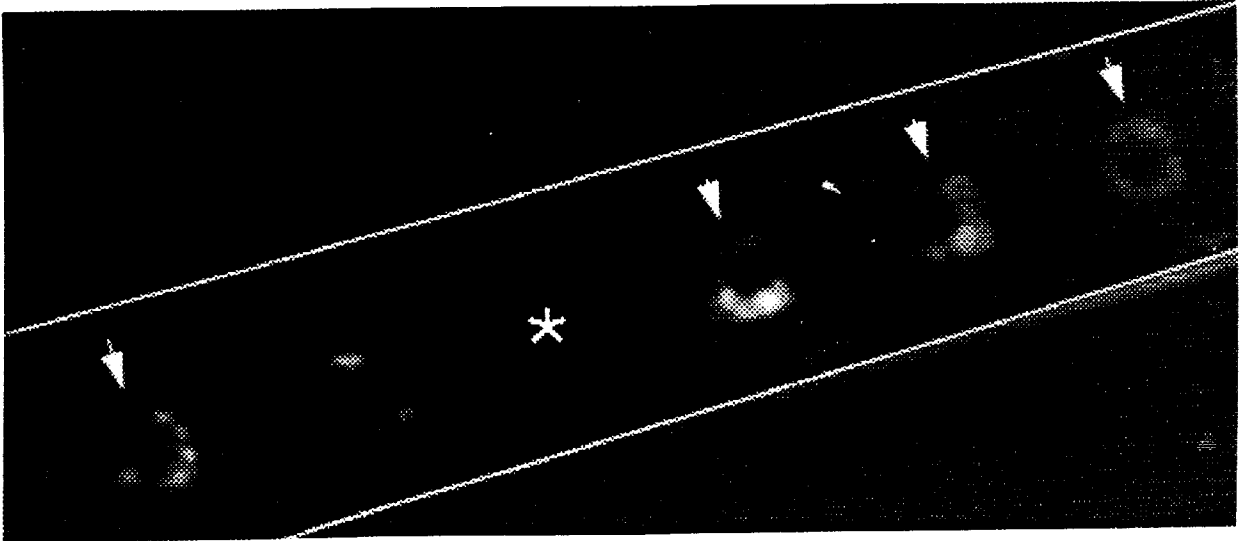
The dead cells likely released the Dil since in wells without cells the staining was absent. Of all six implanted probes, only one growing cell, on the surface of the probe, shown in the figure below, continued to grow after the probe was inserted into the brain.

It is possible that the cause of death is related to the insertion of the probes into the brain since neurons on the handle area which were above the brain surface throughout the procedure continued to grow processes during the subsequent culturing period, as seen in the figure below.
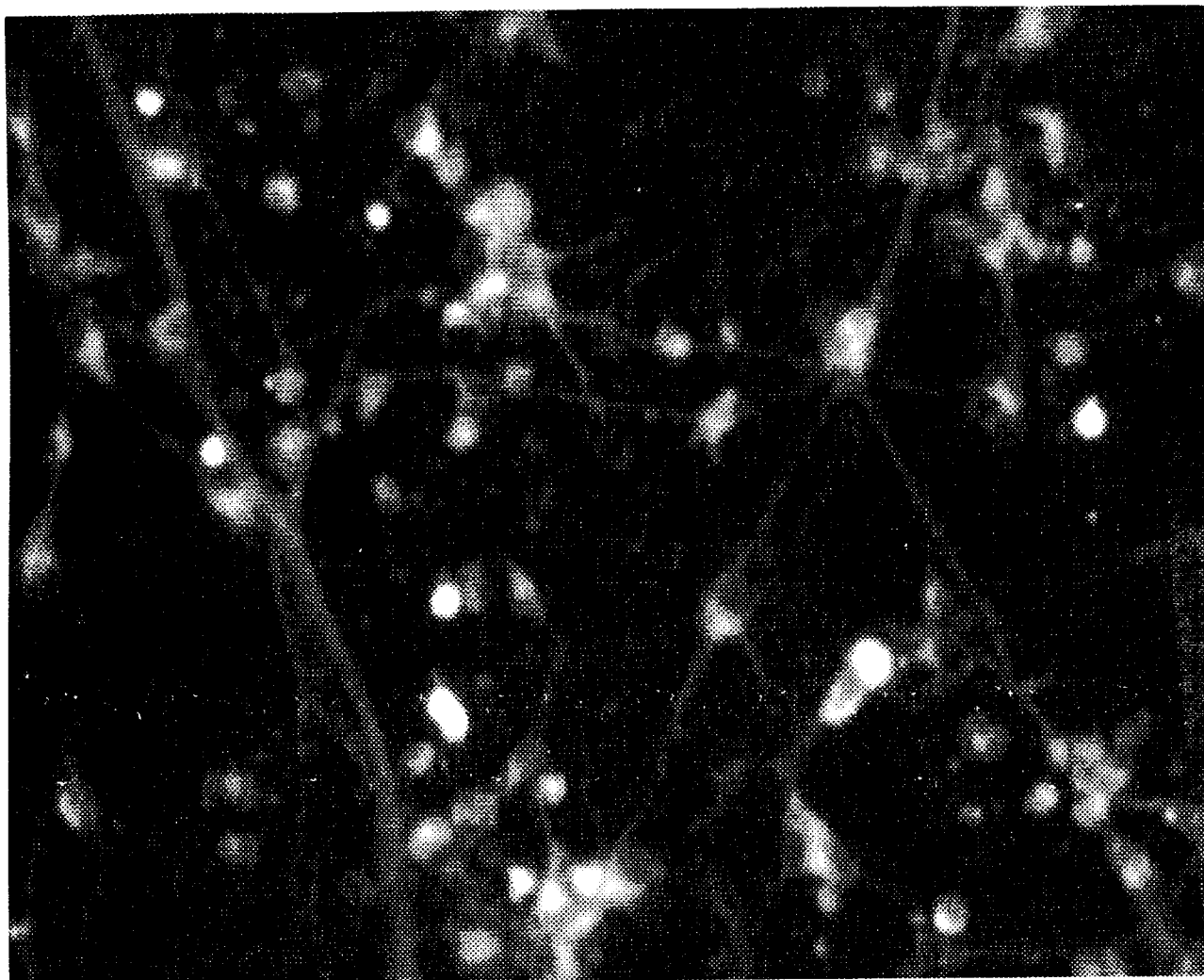
Another photo of a handle area shows both stained septal cells and unstained hippocampal cells growing:
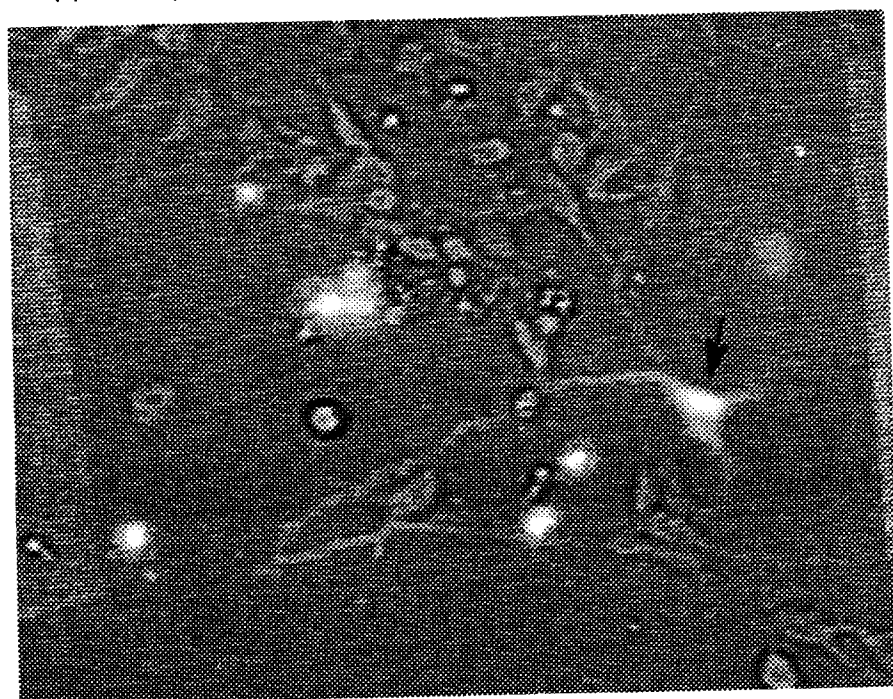
In another set of experiments probes plated with cells were inserted into saline-soaked gelfoam. This procedure removed all processes from the surface of the probe.

The experiments described in this report showed that processes of septal cells are also unable to cross the thing film that covered the surface of the probe after transplantation. The fibrin film and mechanical damage may be the main factors which prevent cell outgrowth after transplantation into the brain.

As has been suggested by our Caltech colleagues, a potential reason of cell damage is due to drying of the silicon probe surface on the air, which process may take only a few seconds. Although we cannot exclude this possibility, the fact that cells at the handle area survived suggests that the main cause of cell death might be factors other than de-humidification. However, in experiments at Caltech, cells on the probe died but those on the handle did not, when the probe was exposed briefly to air. A hypothesis is that the large handle area holds an adequate water layer, while the slim probe does not. During the coming quarter further experiments will need to be done to more clearly determine the cause of cell death.

Slice studies

Our previous neuroprobe design, with cross-shaped silicon-nitride grillwork, had problems with neurons escaping through the corner holes. We recently fabricated dummy neuroprobes (no electrodes) that incorporate the new canopy grillwork. This design features low tunnels (one half-micron tall, by one to four microns wide) through which the neuron's processes must grow. This well geometry has been shown, using neurochips, to be very effective in preventing escape of neurons grown in culture for many days, while allowing processes to grow freely.

Embryonic hippocampal neurons were stained with DiI, as previously described, and placed into wells of the new canopy probes, using a micropipette. As with the canopy neurochips, the new well design was successful at allowing the neurons to grow, with somata remaining trapped in the wells, while extending processes out through the thin tunnels. For example, a number of wells showing nice outgrowth after one day in vitro are shown on the following page. Probe "2:26 w 10" shows a well that had two cells placed in it, and one escaped via the central hole, and continued to grow around the top of the canopy. The remaining cell is trapped, and extends a single lamellepodium through the northeast tunnel. These are fluorescence images made using the 2-photon laser-scanning microscope. A z-series of sections was made of each well, to allow three-dimensional imaging of the cell body and the outgrowth. The images shown are maximum-intensity projections through the z-series, sometimes called 'extended focus' projections.

The 2-Photon microscope was previously shown to be superior to both wide-field fluorescence microscopy, and to confocal microscopy, in terms of dye bleaching and phototoxicity. We have imaged stained neurons with 2-photon time-lapse, every fifteen minutes for over 8 hours, with no adverse affects on cell viability. We have a publication in press in Gene that describes our 2-photon microscope and its benefits.

We have previously made numerous attempts to observe outgrowth from neuroprobes after placing them onto cultured hippocampal slices. In each case, we observed only the remains of dead stained neurons within the wells of

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2:06w15,16
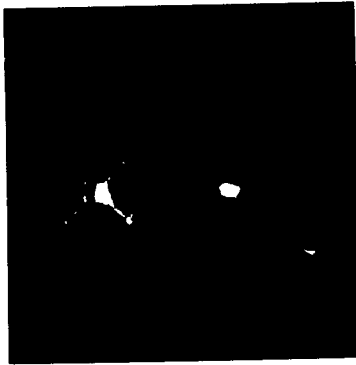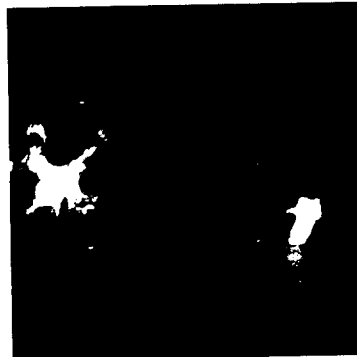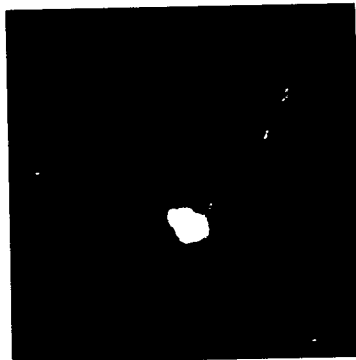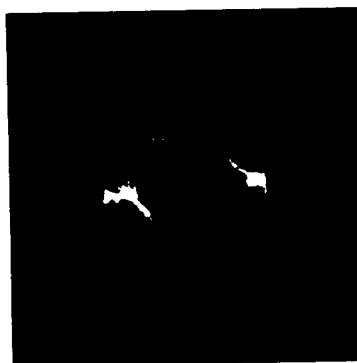
2:26w6,7,8

2:06w4

2:26w10

1:37w10

12:56w2,3

1:37w8

2:06w11,12

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the transferred probe. To investigate the reasons for the death of the transplanted neurons, we first verified that the cultured slices can support the growth of stained neurons seeded directly onto them. As described in the previous quarterly report, we have observed exuberant growth of these transplanted cells. After 3 days "in slico", many fine processes from these neurons can be observed to traverse the entire slice, extending throughout its thickness.

We have now conducted 2-photon time-lapse imaging of neurons growing on the neuroprobes before and after placing the probes onto slices. The "before" time-lapse demonstrated that we could observe the movement of the processes and the dynamics of the extending growth cones. After placing the growing probes onto slices, we observed no further growth of the processes. They were stationary the earliest time (approx. 20 min) they were imaged after the transfer. Clearly, something killed them rapidly during, or soon after the transfer to the slices.

By observing dummy probes dipped in media and rapidly placed under the upright Nomarski microscope, we discovered that the shank of the probe, where the wells are, dries completely within 10 seconds. We did not expect this, since a thin layer of media remains in petri dishes for a least a minute after pouring out the media. We found that the probe shank, being only approximately 150 μm wide by 15 μm thick, retains very little moisture compared to a petri dish, or even compared to the probe handle, and this dries quickly. Thus, we hypothesize that the neurons were dying during the brief (~5 sec) transfer from the culture dish to the slice dish, from either drying or hyperosmolarity.

We verified this by plating unstained cells at a high density onto probes, allowing them to grow in culture for a few days, and then exposing them to air for varying amounts of time. The probes were kept in medium containing 2 μM propidium iodide. This is a non-permeant stain for DNA, and labels only the nuclei of dead cells, whose membrane has been compromised. All neurons with healthy-looking morphology were unlabeled before the air exposure. After as little as 10 sec of air exposure, most of the neurons on the probe shank

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rapidly became labeled with propidium iodide. It was surprisingly difficult to notice any changes in the morphology of the neurons that had been killed by air exposure, until they had several hours to decompose in culture. This explains why we observed stationary processes in the probes that were put onto the slices, and imaged immediately afterwards, and stained debris from dead cells when the probes were observed after several days in slico.

On the following page are two sets of 2-photon images of neurons growing on the old cross-shaped (top) and new canopy (bottom) probes, before and after a 10-sec exposure to the air. Although some processes remain, time-lapse imaging showed no further movement of the visible cells or their processes.

We have successfully solved the drying problem by conducting the probe transfers entirely submerged in medium. We fabricated a 'microdish' from a slice of a blue pipette tip glued to a piece of coverslip with silicone rubber. This can be submerged in the petri dish containing the probe. The slice culture, usually grown with medium only underneath the membrane upon which the hippocampal slices rests, is flooded temporarily with medium. The probe is unglued from the in vitro culture dish and placed in the microdish. The microdish, containing the submerged probe, is submerged in the slice membrane culture dish, and the probe is transferred to the slice without lifting it above the surface of the medium. After the empty microdish is removed, most of the medium is drained away from the top surface of the membrane insert, leaving a small meniscus of medium to wet the probe and slice from above.

In vitro culture dish   Slice membrane-culture dish

**Microdish: underwater probe transfer**
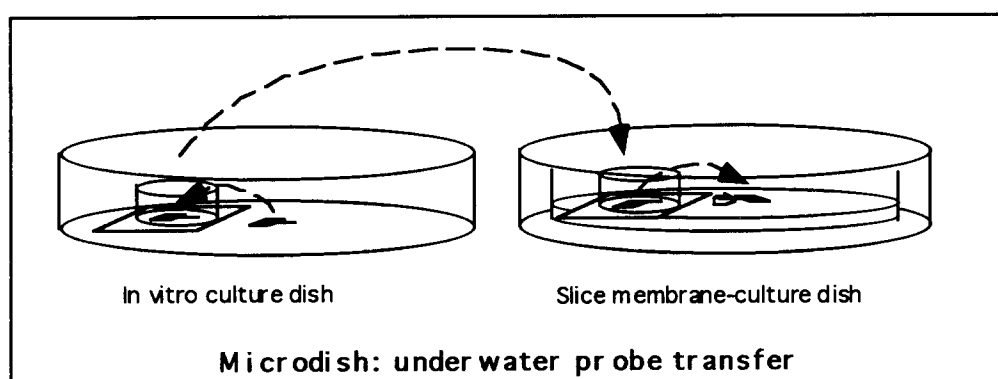
Summary

During the quarter significant progress was made in fabrication, but only after a long slow period during which the microfabrication laboratory was moved to a new building and there were numerous equipment problems to iron out. Successful runs were finally achieved, to produce dummy neuroprobes and complete neurochips. The initial production was in small numbers, but processing bugs appear to have been worked out so that the next runs will be highly efficient.

Soon after production of new complete neurochips a first try was begun to demonstrate recording and stimulation. With good cell growth for one week, the stage was set, but it was discovered that electrical contact with the wells had been lost. The connection of gold epoxy to tinned printed circuit board pads had failed due to decomposition of the tinned layer. A series of experiments were done to pinpoint the area of failure and ultimately to solve the problem. During the coming quarter a new attempt will be made.

Experiments with dummy probes were conducted *in vivo*, in which probes were inserted and then removed after only a few minutes. This study revealed that cell death which had plagued previous implants occurred almost completely on even this short time scale. Various possibilities, including overgrowth by fibrin in the animal or dehydration before insertion need to be evaluated in the coming quarter.

In parallel, dummy probe experiments were conducted by placing loaded probes on cultured slices, "in slico". Death was seen, similar to that *in vivo*, but when a system was devised for keeping the probe immersed while transferring it to the slice the cells survived well, and grew vigorously into the slice. Thus, in these experiments, death could clearly be ascribed to dehydration, but the slice is not of course equivcalent to the rat. A surprising finding in these studies was that cells growing many processes through tunnels withdrew them and escaped out the central hole of the well during a two day period when it was engulfed by overgrowing slice cells. They remained on the top of the canopy, growing happily. This phenomenon will be studied in the coming quarter, after a longer period of outgrowth through the tunnels.

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Before

After

17
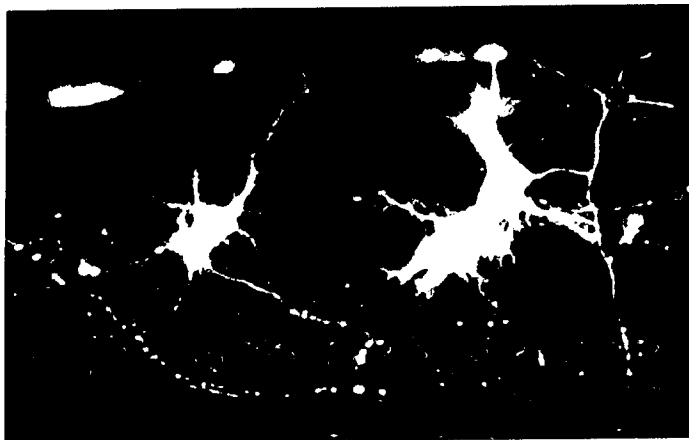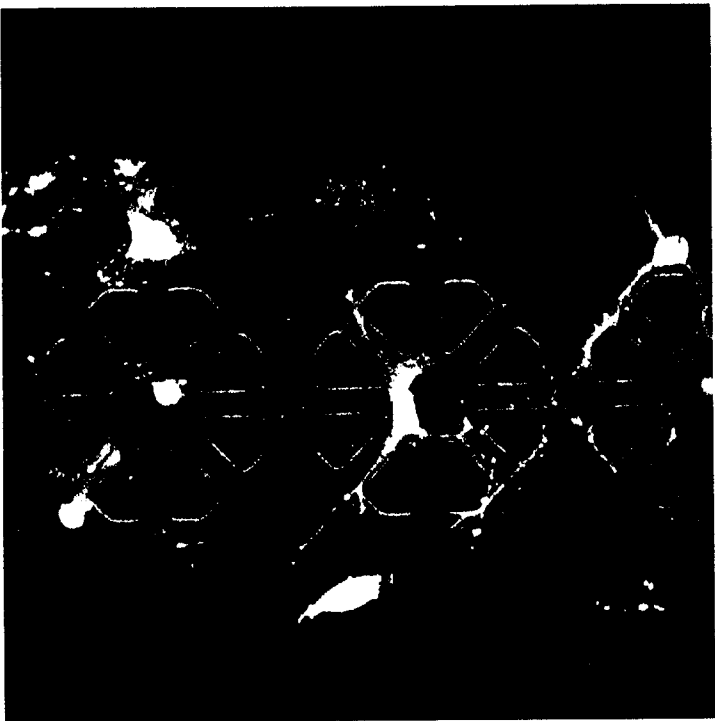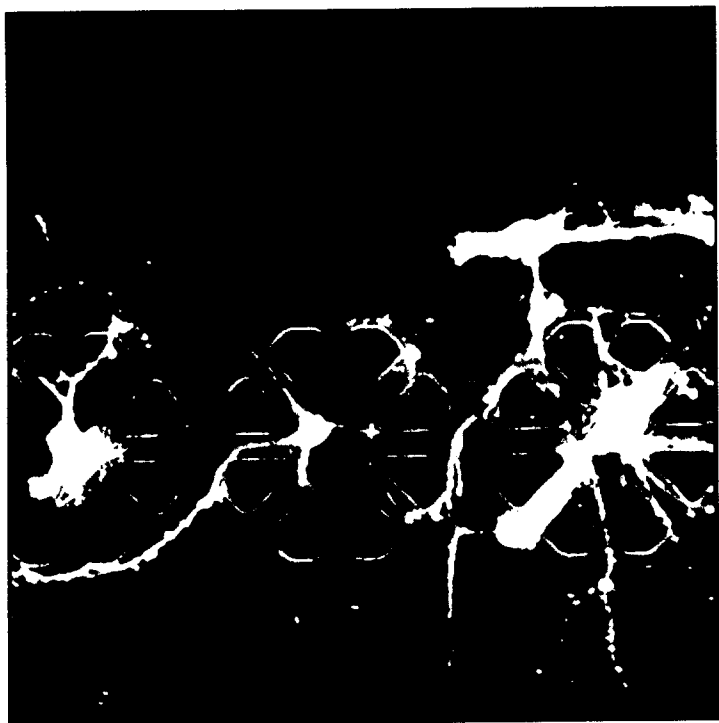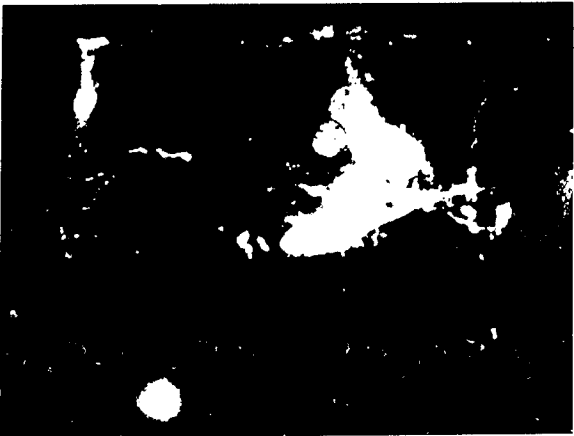
After a day or two, the probe shank sinks through the thickness of the slice (~200 μm) and is completely covered by slice neurons and glia that have migrated over the probe.

We have observed excellent survival of probe neurons transferred using the microdish. After several days in slico, long processes extending hundreds of microns from the probe can be observed at all levels through the thickness of the slice. However, we are now faced with a serious problem that appears to be peculiar to the cultured slices: The probe neurons escape through the center holes of the canopy grillwork. Three days after transferring the probes loaded with DiI-stained neurons to slices, all of the labeled neurons' somata were found near the wells, on the surface of the probe or a few tens of microns above the probe. Of four probes transferred, with 34 living neurons in wells before the transfer, all had escaped.

The top left-hand panels of the figures on the following three pages show 2-photon images of outgrowth from canopy probes one day after placing the neurons into the wells. The probes were placed onto slices minutes after the images were made, using the microdish, cultured for 3 days, and imaged with the 2-photon microscope, in either 2-photon or confocal mode (visible excitation). The top right panels show vigorous outgrowth from these neurons, which extended several hundred microns further into the slice in all cases. In the bottom panels, reflectance or transmission images reveal the location of the wells that previously housed the neurons. (The thin layer of silicon nitride at the base of the well is transparent.) The overlay images in the lower right show that the neuron somata have all escaped, presumably through the central holes.

It should be emphasizeed that the neuron locations above the wells were verified by 3D projections of a z-series of 2-photon images. Apparently, the presence of slice cells above the central hole, and maybe growing into it, causes the neurons in the wells to retract the processes that were growing through the tunnels, and migrate out through the hole. We plan to grow the probes longer before putting them onto the slices, so that the processes through the tunnels will be more firmly established, making it harder for the cell bodies to escape from the wells when the probes are put onto slices.
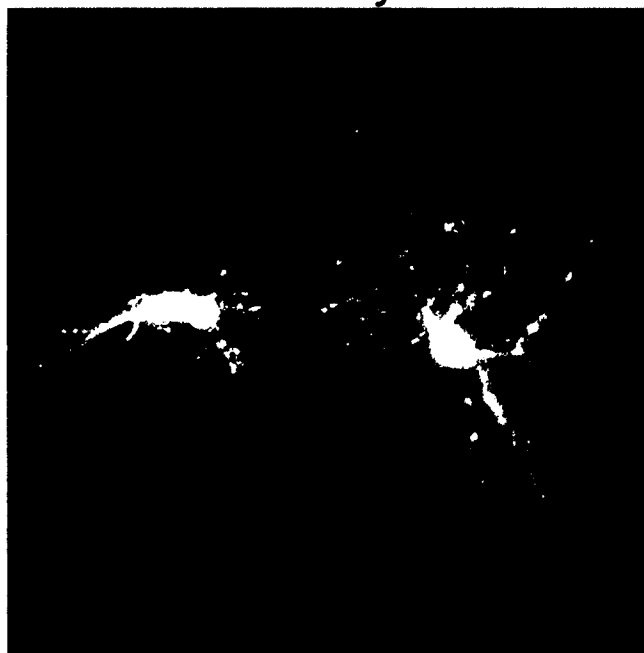
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2:06w4

2:06w4,5 (neurons above wells)

Reflectance image of the same field,
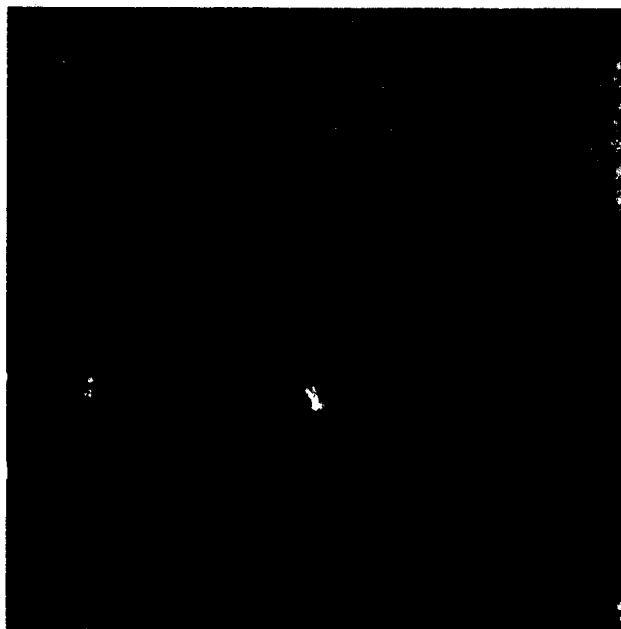seen through unlabeled slice neurons. Overlay of both images

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2:06w15,16 in vitro

After 3 days in slico

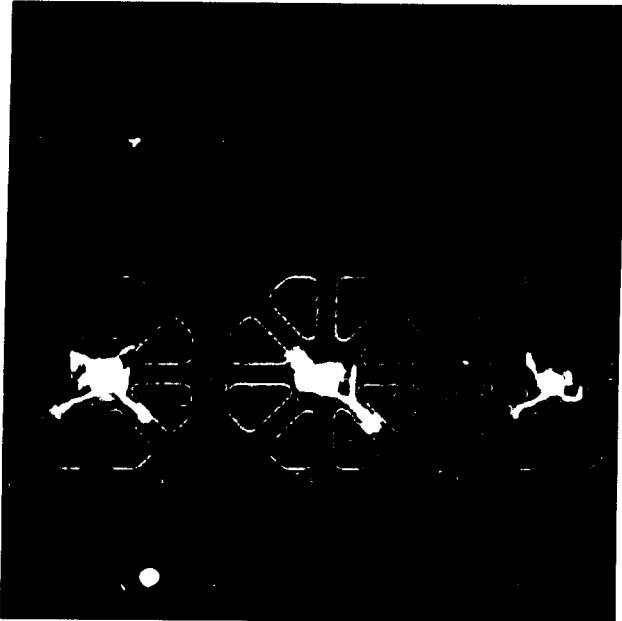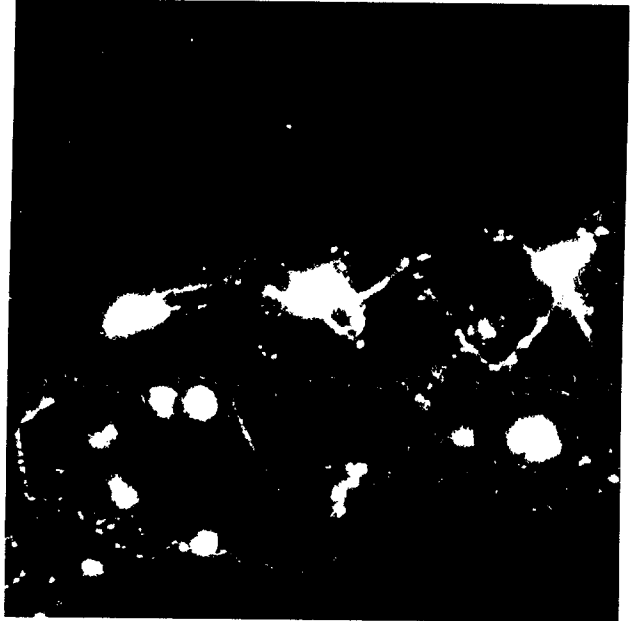## Transmission and overlay images

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2:26w6,7,8 in vitro

After 3 days in slico:
All escaped.

Transmission and overlay images